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13. ABSTRACT (Maximum 200 Words)
The breast cancer susceptibility gene BRCA1 is mutated in many cases of familial breast and ovarian cancer. BRCA1 is phosphorylated from S to M phase of the cell cycle, as well as in response to DNA damage, and a hypophosphorylated form of BRCA1 is found at the centrosome during M phase of the cell cycle. Phosphorylation may play an important role in the regulation of BRCA1 function. We have performed a yeast two-hybrid study in order to identify proteins that interact with exon11 of BRCA1. Among the proteins identified in the screen was Protein Phosphatase 1 (PP1), a serine threonine phosphatase. PP1 and BRCA1 co-immunoprecipitate both *in vitro* and *in vivo*, and a GST pull down assay has identified the region within BRCA1 that is involved in the interaction. Colocalization studies of the two proteins provide further evidence for an interaction of BRCA1 and PP1 within the nucleus of the cell. Mutational analysis has identified 2 sequence alterations in PP1α, and expression analysis of PP1□ mRNA has been performed. Studies also show that PP1α dephosphorylates BRCA1 *in vitro*. The interaction of PP1 and BRCA1 represents a potentially significant interaction that could have an affect on the function of BRCA1.

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INTRODUCTION

The breast cancer susceptibility gene, BRCA1, is involved in the development of a large proportion of familial breast and ovarian cancers, and may also play a role in the development of sporadic breast cancer.

BRCA1 interacts with a large number of proteins, and may be functioning as a scaffold protein to coordinate DNA repair, transactivation and centrosome separation or maturation. The phosphorylation levels of BRCA1 change according to the cell cycle, with a hypophosphorylated version of BRCA1 found during G1/S. BRCA1 is then phosphorylated from S-G2/M and hypophosphorylated form of BRCA1 is present at the centrosome during mitosis. The phosphorylation status of BRCA1 could have important consequences on its ability to behave as a scaffold protein to effect repairs after DNA damage, or could have an affect on its ability to coordinate cell division.

We have performed a yeast two hybrid assay using exon11 of BRCA1 to screen interacting proteins from a human mammary gland cDNA library and have identified Protein phosphatase 1 as a BRCA1 interacting protein. A number of putative phosphorylation sites are located within exon 11 including Ser308, phosphorylated by Aurora A kinase at the onset of the G2/M transition and Ser 988, phosphorylated by ChkII (CDS1) after exposure to DNA damaging agents. Aurora A localizes to the centrosome at centriole duplication, and cells that overexpress Aurora A often have polyploidy as well as centrosome amplification, characteristics of breast tumors containing mutations in BRCA1. Aurora A phosphorylates BRCA1 at Ser 308 (ref 1), and increased phosphorylation of this residue is observed in early M phase of the cell cycle; however, this phosphorylation is abolished with ionizing radiation. These studies suggest that phosphorylation of Ser308 may have important consequences for the function of BRCA1 at the centrosomes, therefore the interaction of PP1 and BRCA1 and subsequent dephosphorylation of Ser308 by PP1 may be an important event ensuring a proper G2/M cell cycle checkpoint and centrosome separation occurs. This project hopes to further characterize the interaction of BRCA1 and PP1, and to identify the potential significance of this interaction in the development of breast cancer.

BODY

SPECIFIC AIM 1: Identification of the region of interaction between BRCA1 and Protein Phosphatase 1 (PP1)

Task 1: As reported in the mid-term review, task 1 has been completed. BRCA1 was shown to interact with PP1 both in an *in vitro* GST pull down experiment and in an *in vivo* co-immunoprecipitation experiment and the region of BRCA1 that interacts with PP1 was identified to be BR-4 (a.a. 758-1064). Additionally, there is a PP1 interacting domain KVTF within BR-4. Mutating Val to Ala or Phe to Ala disrupts the interaction between BR-4 and PP1. The degree to which the interaction has been disrupted has now been quantified using a BioRad Gel Documentation system (model and program). The Val-Ala alteration disrupts the interaction between PP1 and BR-4 by approximately 80%, and the Phe-Ala alteration results in a 70% disruption of the interaction compared to the interaction of wild-type BR-4 with PP1 (Figure 1). These alterations are also being studied to determine their affect on the ability of PP1 to dephosphorylate BRCA1 by PP1.

A second putative PP1 interacting domain can be found in BR-2 (FCNKSK (aa304 to 309)). Interestingly this sequence overlaps with the Serine 308 phosphorylated by Aurora A kinase. Additional studies will be performed to identify if there are conditions under which BR-2 and PP1 interact.

Task 2 and 3: This task has not been completed, as all PP1 constructs that have been made are insoluble in bacteria.

Specific Aim 2: Analysis of the cellular and temporal localization patterns of BRCA1 and PP1

Task 1 and 2: As previously described in the mid-term report, colocalization of BRCA1 and PP1 was observed in NIH-3T3 cells that had been synchronized using double thymidine selection. NIH-3T3 cells were used as the conditions for synchronization were known and they are an optimal shape for performing immunofluoresence; therefore they were chosen as the initial cells used to determine if the antibodies to PP1 and BRCA1 would be suitable for immunofluoresence. BRCA1 and PP1 colocalized and the appearance of the chromosomes suggested that the cells were in early M phase. The antibodies used were to endogenous BRCA1 and to endogenous PP1; however, the specific isoform of PP1 that interacted with BRCA1 could not be determined. Stable cell lines of Flag-epitope tagged PP1α, β or γ were constructed in SKOV3 human ovarian cancer cells in order to distinguish the various roles of the PP1 isoforms; however, although these cells were successfully transfected with the G418 resistant pCMV-FLAG-PP1 vector, they did not express or had very low levels of expression of PP1. This may be due to the growth suppressive functions of PP1, allowing for a selective advantage to cells that maintained the vector, but had low or nonexistent expression of PP1. Therefore, antibodies specific to each of the PP1 isoforms will be optimized for immunofluorescence in order to determine if PP1α and BRCA1 are colocalizing at the centrosomes during the onset of M phase. Additional antibodies found at the centrosome such as γ-tubulin will be used to confirm the cellular localization of BRCA1 and PP1. Furthermore, it will be determined if Aurora a kinase also colocalizes with BRCA1 and PP1 at the centrosomes at the onset of M phase.

Task 3: As task 3 was to determine the effect of irradiation on the localization of proteins, and optimization of this experiment is ongoing, task 3 is not yet completed.

SPECIFIC AIM 3: Mutational Analysis of BRCA1 and PP1

Task 1 and 2: As described previously in the mid-term report, task 1 and 2 have been completed and none of the tumor-associated missense mutations in BRCA1 that were tested had an affect on the interaction of BRCA1 and PP1. Other missense mutations studied were in the PP1 interacting domain within BR-4 and did disrupt the interaction (see specific aim 1), but these sequence alterations within the BR-4 region are not associated with tumors. No further missense mutations have been analyzed (*Tasks 3 & 4*)

Task 5: Single Strand Conformation Polymorphism analysis (SSCP) had previously been performed on PP1β, and no sequence alterations were identified (please see mid-term report). SSCP for PP1α has been performed on DNA from 130 sporadic breast tumors. Two tumors contained tyrosine-tyrosine polymorphisms within exon 6 of PP1α and 1

tumor contained an Ala-Arg alteration (Figure 2). Additionally, 1 Ala-Thr alteration was observed in exon7 of PP1α (Figure 2). Control DNA from non-cancerous patients will be analyzed to determine if these alterations are present in the general population and if they are not identified, the sequence alterations will be cloned into PP1α to determine if they have an affect on the interaction of PP1 and BRCA1 or the ability of PP1 to dephosphorylate BRCA1 (*Task 6 and 7*).

Task 8: Real-time PCR (ABI Biosystems) has been performed on PP1α using mRNA from 42 sporadic breast tumors and 8 tumor-normal pairs to determine PP1α expression in these tumors. Real-Time PCR was also done in the same PCR tube for the house keeping gene PGK (phosphoglycerate kinase) in order to control for differences in mRNA levels from tumor to tumor. A wide range of expression was observed in PP1α when comparing sporadic tumors (Figure 3a). These expression results will now be correlated with the expression of other genes that have been previously analyzed in our lab using microarrays. There was a trend of decreased expression of PP1α when comparing tumor mRNA to normal mRNA from the same patient; however, the difference in expression was not great enough to be statistically significant (Figure 3b).

SPECIFIC AIM 4: Study of the association of BRCA1 and PP1 with other BRCA1 associated proteins

Tasks 1 and 2: As the conditions required to analyze the colocalization of BRCA1 and PP1 are being further optimized, these tasks have not yet been performed. Upon successful optimization of the colocalization conditions, the interaction of BRCA1 with PP1 at the centrosomes, and with Aurora A kinase during G2-M phase of the cell cycle will be looked at.

ADDITIONAL EXPERIMENTS PERFORMED NOT OUTLINED IN THE GRANT:

Dephosphorylation of BRCA1 by PP1α

Dephosphorylation studies have been performed to determine if PP1 is able to dephosphorylate BRCA1 after phosphorylation by ChkII kinase (ref 2). GST fused BR-4 and GST-fused ChkII were mixed and an *in vitro* phosphorylation assay was performed. The beads were precipitated and washed, followed by the addition of recombinant PP1α. PP1α was able to dephosphorylate BR-4 after phosphorylation by ChkII. The affect of the Val-Ala and Phe-Ala alterations within the KVTF PP1 interacting domain found in BR-4 on the ability of PP1 to dephosphorylate BRCA1 will be determined. Also, the ability of PP1 to dephosphorylate Ser308 on BRCA1 after phosphorylation by Aurora A kinase will also be determined (Figure 4).

KEY RESEARCH ACCOMPLISHMENTS

- Quantified the affect of Val-Ala and Phe-Ala sequence alterations within the PP1 interacting domain KVTF found in BR-4 on the interaction of BRCA1 and PP1. The Val-Ala change decreases the interaction of the two proteins by 80% and the Phe-Ala alteration decreases the interaction by 70% when compared to the interaction of wild-type BR-4 with PP1.

- Determined that the creation of stable PP1α, β or γ cell lines will not be possible due to the growth suppressive effects of PP1 on cells, therefore antibodies to the endogenous PP1 isoforms will be optimized.

- 130 sporadic breast tumors were analyzed for sequence alterations within PP1α using SSCP. A Tyr-Tyr polymorphism within exon6 of PP1α was observed, as was an Ala-Arg sequence change. Exon7 of PP1α contained an Ala-Thr alteration.

- Real-time PCR was performed on mRNA from 42 sporadic breast cancer cases and varied levels of expression of PP1α were observed when comparing tumor to tumor. These levels will now be compared with the expression of other genes within the tumor previously tested in our lab using microarrays. Real-Time PCR was also performed on 8 tumor-normal pairs of mRNA and a trend of decreased expression was observed in mRNA from tumors compared to mRNA from normal tissue; however this result was not statistically significant

- Dephosphorylation studies have been done to determine if PP1 can dephosphorylate BRCA1. PP1α dephosphorylated a region of BRCA1 (BR-4) that was phosphorylated by ChkII kinase. The affect of the V-A or F-A alterations on the ability of PP1 to dephosphorylate BRCA1 will be studied, as well as the ability of PP1 to dephosphorylate BRCA1 after phosphorylation by Aurora A kinase will also be determined.

REPORTABLE OUTCOMES:

Abstracts presented:

Winter, S.L., Andrulis, I.L. "Characterization of the Interaction of BRCA1 and Protein Phosphatase 1" presented at the 12th International Conference on Second Messengers and Phosphoproteins, August 3-7, 2004.

Training Accomplishments:

The PI attends weekly lab meetings to discuss both her own and others work in the lab. In addition, weekly journal clubs discussing key research publications are attended. The PI has presented her work at the departmental monthly Cancer Genetics meeting. Furthermore, posters have been presented at the yearly institute retreat, and will also be presented at the 12th International Conference on Second Messengers and Phosphoproteins in August 2004. All course work required for the completion of her thesis has been done; including courses on Human Genetics, Proteomics and Genomics, Protein Structure and Function and Cancer. Yearly meetings with the thesis committee members have occurred, and work is now proceeding towards finishing up final experiments and writing results in the form of papers for journals as well as the PhD thesis. The PI has also instructed undergraduates in a 4th year laboratory course, as well as a 3rd year course designed to teach students how to analyze scientific papers, and the

PI is supervising a summer student working on another aspect of the PI's project. The opportunity to teach undergraduates provides valuable training for future work that might include teaching at a university level.

CONCLUSIONS:

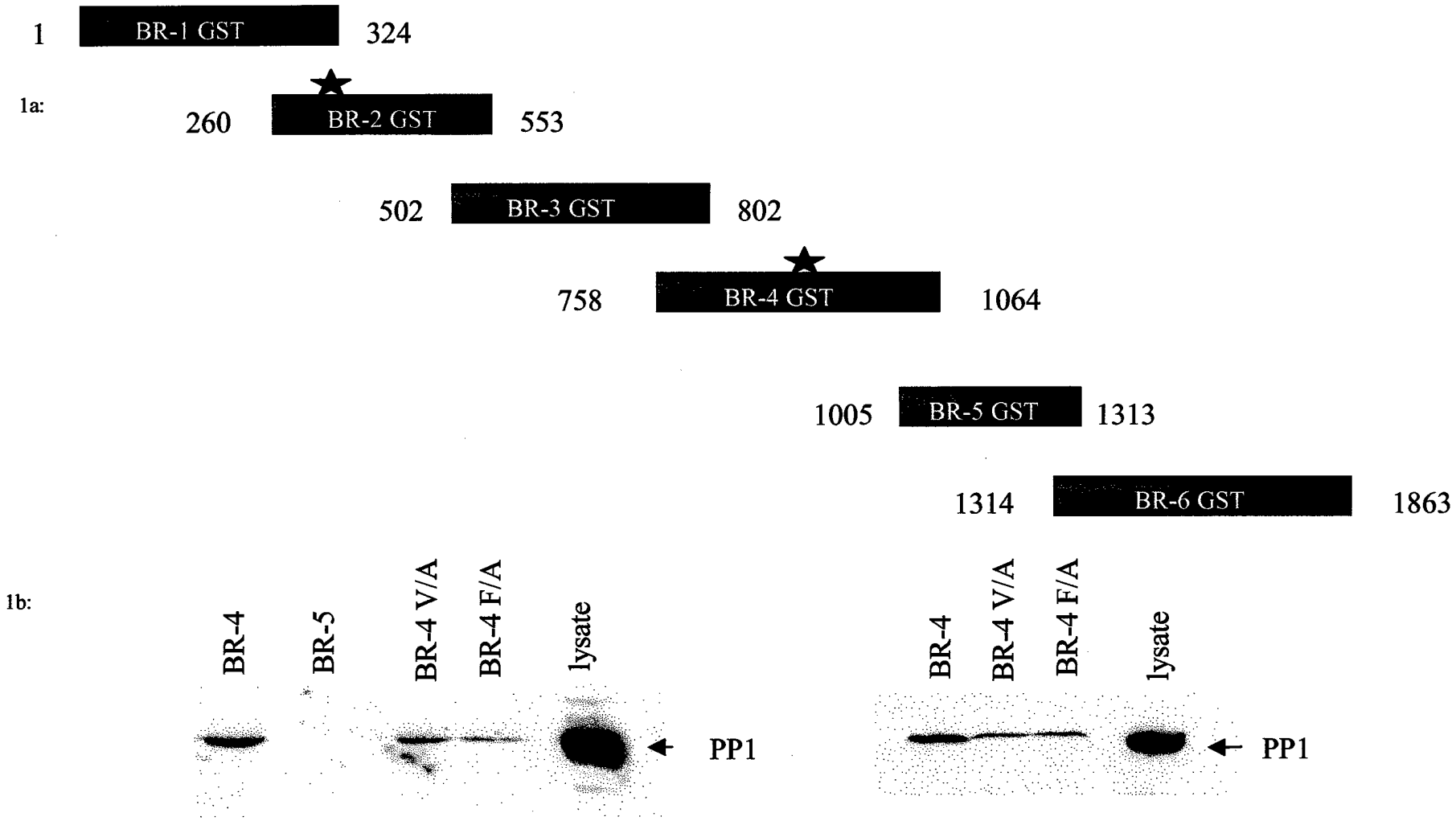
Co-immunoprecipitation, colocalization and GST-pull down studies confirm the interaction of PP1 with BRCA1. Further studies with synchronized cells and colocalization studies with proteins known to be localized to the centrosome during mitosis should help to clarify the role of this interaction with BRCA1 and PP1. Expression studies indicate that PP1α may be decreased in breast tumors. This, coupled with potential interaction of BRCA1 and PP1 at the centrosomes suggests that this interaction may play a role during centrosomal separation or maturation. Interestingly, Aurora A kinase is amplified in a number of cancers. Decreased expression of PP1 could have the same affect, resulting in abnormal centrosomal separation. Disruption of this interaction could therefore result in altered chromosome separation and aneuploidy, a phenotype commonly observed in breast cancers with BRCA1 mutations.

REFERENCES:

1. Ouchi M, Fujiuchi N, Sasai K, Katayama H, Minamishima YA, Ongusaha PP, Deng C, Sen S, Lee SW, Ouchi T. 2004. BRCA1 phosphorylation by Aurora-A in the regulation of G2 to M transition. J Biol Chem. 2004 May 7;**279**(19):19643-8. **Epub 2004 Feb 27**

2. Liu, Y., D.M. Virshup, R.L. White, and L.C. Hsu. 2002. Regulation of BRCA1 phosphorylation by interaction with protein phosphatase 1 alpha. Cancer Research **62**:6357-61.

Fig. 1 The affect of altering the PP1 interaction domain found in BRCA1 on the interaction of BRCA1 and PP1



A: 7 vectors were constructed with fragments of BRCA1 fused in frame to the GST epitope. There is a putative PP1 interacting domain KVTF (898-901) found within BR-4. There is also a putative interacting domain in BR-2, overlapping Ser 308, which is phosphorylated by Aurora-A kinase (FCNKSK)

B: The GST fragment BR4 V-A binds PP1 with 0.31x the intensity, and F-A binds PP1 with 0.22x the intensity compared to WT BR4, when 0.5µg of GST bound protein is incubated with an equal amount of cell lysate.

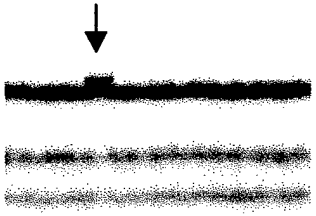
Figure 2:

SSCP Results

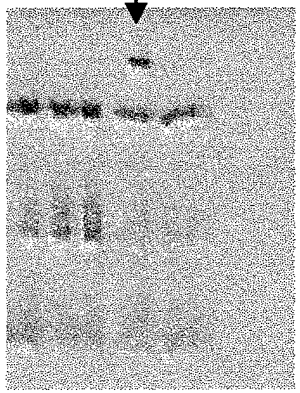
130 sporadic breast tumour samples were analyzed for sequence alterations

Exon 2:	No shifts
Exon 3:	No shifts
Exon 4:	Intron shifts: 8 tumours: g-a, 5 tumours: g-a + c-g, 1 tumour: insertion adjacent to exon (splicing?)
Exon 5:	No shifts
Exon 6:	2 shifts: Tyr-Tyr 1shift Ala-Arg
Exon 7:	1 shift: Ala-Thr

Exon 6 shift



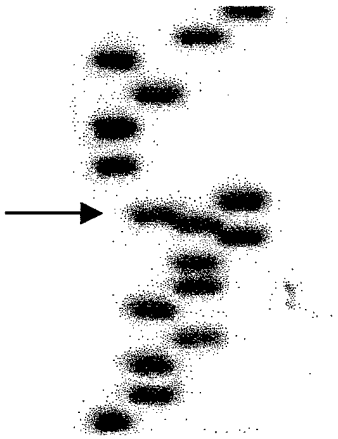
Exon 7 shift



Exon 6 sequence of shift

A C G T

gct-cgt
Ala-Arg



Exon 7 sequence of shift

A C G T A C G T

gcc-acc
Ala-Thr

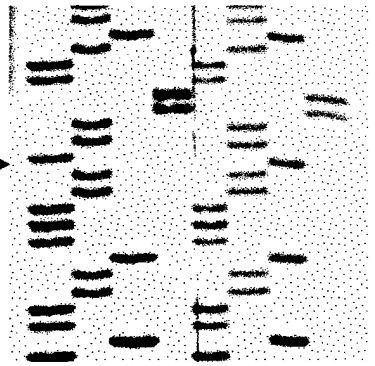
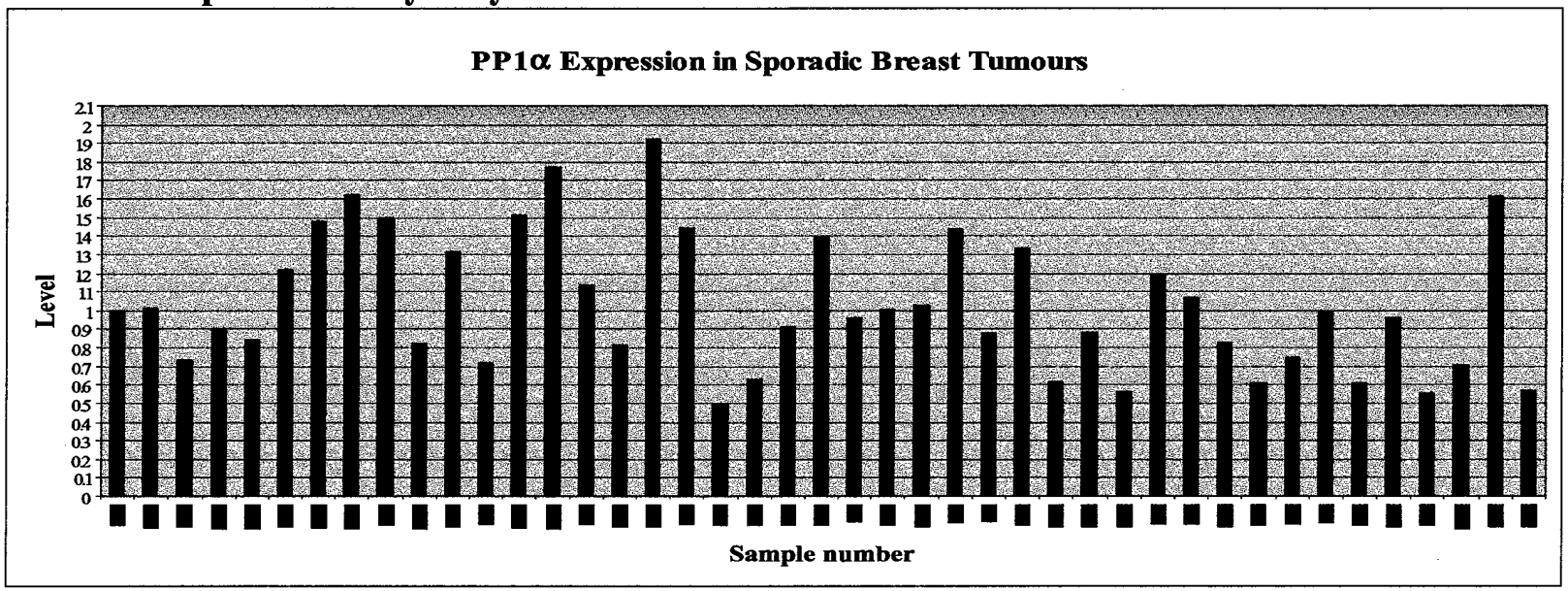
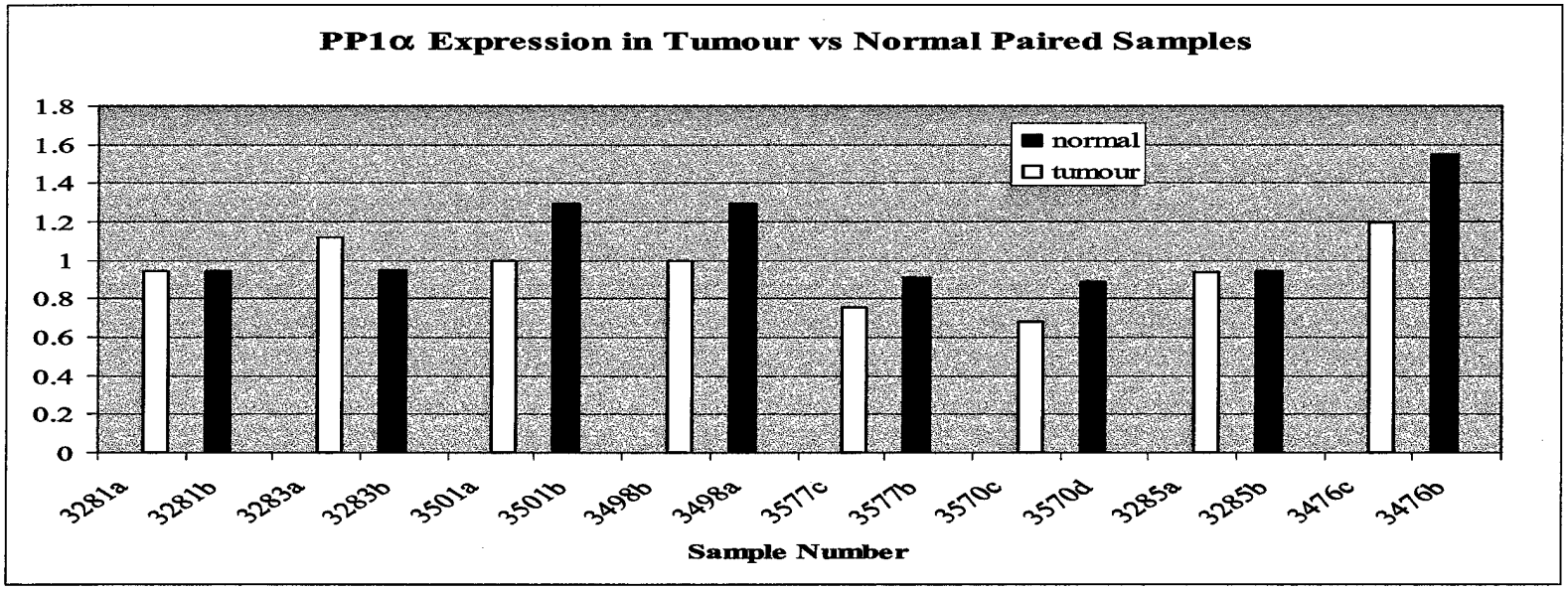


Figure 3: PP1α expression analysis by Real-Time PCR:

3a

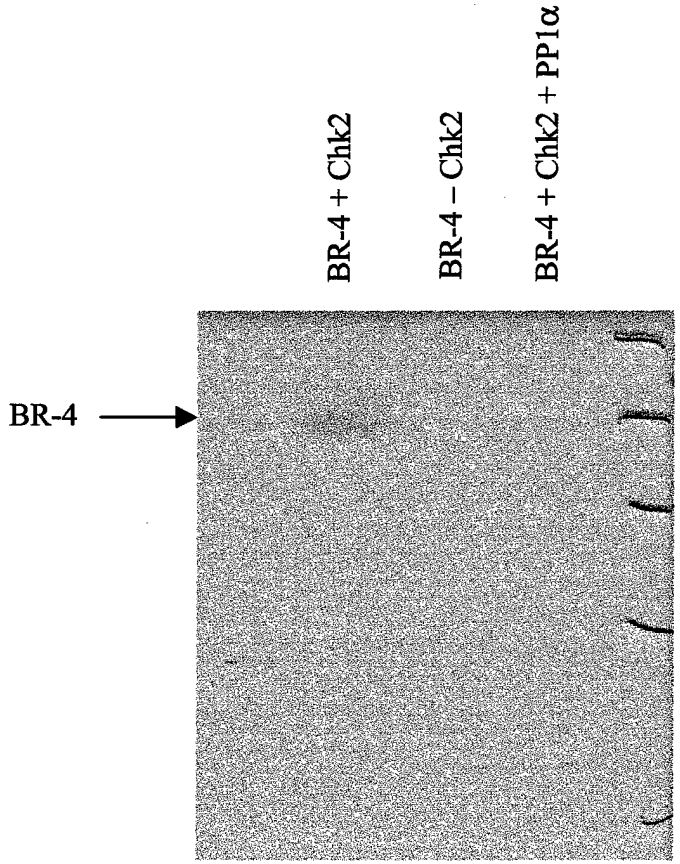


3b



PP1α expression levels were measured by Real-Time PCR either comparing levels between sporadic breast tumors (3a), or levels compared between tumor and normal tissue paired samples (3b).

Figure 4: PP1α dephosphorylates BR-4 at Chk2 phosphorylation site (Ser 988)



1μg of BR-4 GST is mixed with 1μg of Chk2-GST, and a kinase assay is performed with p^{32}ATP. Recombinant PP1α is then added to dephosphorylate Br-4.